Temperature-responsive genetic loci in the plant pathogen *Pseudomonas syringae* pv. *glycinea*

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Plant-pathogenic bacteria may sense variations in environmental factors, such as temperature, to adapt to plant-associated habitats during pathogenesis or epiphytic growth. The bacterial blight pathogen of soybean, *Pseudomonas syringae* pv. *glycinea* PG4180, preferentially produces the phytotoxin coronatine at 18 °C and infects the host plant under conditions of low temperature and high humidity. A miniTn5-based promoterless glucuronidase (*uidA*) reporter gene was used to identify genetic loci of PG4180 preferentially expressed at 18 or 28 °C. Out of 7500 transposon mutants, 61 showed thermoregulated *uidA* expression as determined by a three-step screening procedure. Two-thirds of these mutants showed an increased reporter gene expression at 18 °C whilst the remainder exhibited higher *uidA* expression at 28 °C. MiniTn5-*uidA* insertion loci from these mutants were subcloned and their nucleotide sequences were determined. Several of the mutants induced at 18 °C contained the miniTn5-*uidA* insertion within the 32.8 kb coronatine biosynthetic gene cluster. Among the other mutants with increased *uidA* expression at 18 °C, insertions were found in genes encoding formaldehyde dehydrogenase, short-chain dehydrogenase and mannuronan C-5-epimerase, in a plasmid-borne replication protein, and in the *hrpT* locus, involved in pathogenicity of *P. syringae*. Among the mutants induced at 28 °C, insertions disrupted loci with similarities to a repressor of conjugal plasmid transfer, UV resistance determinants, an isoflavanoid-degrading enzyme, a HU-like DNA-binding protein, two additional regulatory proteins, a homologue of bacterial adhesins, transport proteins, LPS synthesis enzymes and two proteases. Genetic loci from 13 mutants did not show significant similarities to any database entries. Results of plant inoculations showed that three of the mutants tested were inhibited in symptom development and *in planta* multiplication rates. Temperature-shift experiments suggested that all of the identified loci showed a rather slow induction of expression upon change of temperature.

Keywords: temperature, differential gene expression, thermoadaptation, phytotoxin, plant pathogen

INTRODUCTION

In contrast to thermoregulation of virulence factors in

Abbreviations: COR, coronatine; GUS, β -glucuronidase; HR, hypersensitive reaction; X-Gluc, 5-bromo-4-chloro-3-indolyl glucuronide.

The GenBank accession numbers for the nucleotide sequences of mutants 560, 561, 562, 563, 564, 568, 570, 574, 590, 591, 593, 596, 599, 601, 605, 608, 613, 617, 618, 626 and 632 determined in this work are AF274322–AF274342, respectively.

human and animal pathogens (Mekalanos, 1992; Wharam *et al.*, 1995; Hurme & Rhen, 1998), little is known about the influence of temperature on secondary metabolism and host-adaptive processes in phytopathogenic bacteria. In part, this lack of information may be explained by the fact that plants are thought of as poikilothermic organisms that do not represent habitats with constant temperature conditions for the invading pathogens. However, effects of tem-

perature on secondary metabolism, virulence factor production and infection efficiency of phytopathogens have been reported in a number of cases (Ullrich *et al.*, 1995; Banta *et al.*, 1998; Goss, 1970; Hugouvieux-Cotte-Pattat *et al.*, 1992; Jin *et al.*, 1993; Lanham *et al.*, 1991; Rowley *et al.*, 1993; Fullner & Nester, 1996; Budde & Ullrich, 2000).

The bacterial blight pathogen *Pseudomonas syringae* pv. *glycinea* PG4180 infects soybean plants (*Glycine max*), inducing typical leaf spot symptoms characterized by water-soaked regions which soon develop into necrotic lesions surrounded by chlorotic haloes. Like most representatives of *P. syringae*, the pathovar *glycinea* is an opportunistic pathogen that requires water films and aerosol formation for infection of plant tissues via the plant's stomata or open wounds. The symptoms of bacterial blight are most severe during or following periods of cold, humid weather conditions (Dunleavy, 1988).

P. syringae pv. *glycinea* produces the chlorosis-inducing polyketide phytotoxin coronatine (COR) (Bender *et al.*, 1991). COR-deficient mutants of *P. syringae* pvs *tomato*, *glycinea* and *maculicola* were shown to be severely impaired in virulence on tomato, soybean and Chinese cabbage plants, respectively (Bender *et al.*, 1987; Mittal & Davis, 1995; Budde & Ullrich, 2000; Tamura *et al.*, 1998). PG4180 synthesizes high levels of COR at 18 °C, whereas no detectable toxin is produced at 14 or 28 °C (Palmer & Bender, 1993; Budde *et al.*, 1998). Previously, we reported that the temperature-sensitive transcriptional activation of three promoters within the 32.8 kb plasmid-borne COR biosynthetic gene cluster might explain thermoregulation of COR biosynthesis (Ullrich & Bender, 1994; Liyanage *et al.*, 1995; Ullrich *et al.*, 1995). Two of these promoters control the expression of biosynthetic operons whilst the third is located upstream of *corS*, a gene encoding the histidine protein kinase CorS which, together with the two response regulators CorR and CorP, forms a temperature-dependent two-component regulatory system (Ullrich *et al.*, 1995; Budde *et al.*, 1998).

The higher infection rate and stronger symptom development at lower temperatures prompted us to investigate whether COR biosynthesis represented the only temperature-controlled factor of *P. syringae* pv. *glycinea*. A comprehensive search for additional thermoregulated genetic loci was conducted to better understand which factors might potentially be involved in the response of *P. syringae* to minor temperature shifts possibly taking place during micro-climatic changes on the leaf surface. Such temperature fluctuations are often associated with alterations in humidity. Since *P. syringae* pv. *glycinea* requires high humidity for plant infection, low temperatures might be a signal for the epiphytically growing pathogen to start an effective invasion process.

In this study, *P. syringae* pv. *glycinea* PG4180 was randomly mutagenized using the transposon miniTn5 harbouring a promoterless β -glucuronidase (GUS) gene as the reporter system (Wilson *et al.*, 1995). Mutants

were screened for differential expression of GUS at 18 and 28 °C. Following a three-step screening procedure, interesting mutants were differentiated with respect to insertions inside or outside the COR biosynthetic gene cluster. Insertion loci of PG4180 mutants not affected in phytotoxin synthesis were subcloned and sequenced, allowing the identification of additional thermoreponsive genetic loci of *P. syringae* pv. *glycinea* PG4180.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were maintained on mannitol-glutamate (MG) minimal medium (Keane *et al.*, 1970) at 28 °C. Prior to temperature-dependent growth studies, a single colony from a 4-d-old MG agar plate was resuspended in 5 ml King's B medium (King *et al.*, 1954) and incubated overnight on a rotary shaker at 280 r.p.m. and 28 °C. Aliquots of 100 μ l of the overnight culture served as inoculum for 10 ml HSC medium (Palmer & Bender, 1993) in test tubes which were incubated under identical growth conditions as described for the overnight culture but at either 18 or 28 °C. Bacterial growth was monitored by measuring the optical density at 600 nm. After 24–48 h, bacterial cells were harvested by centrifugation and supernatants were filter-sterilized. Cells were lysed by sonication. Protein contents of cell lysates were determined by the Bradford assay (Sambrook *et al.*, 1989). *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) was used as a host for DNA cloning and was cultured in 5 ml Luria–Bertani medium in test tubes at 37 °C. The following antibiotics were added to the media when needed (in μ g ml⁻¹): ampicillin, 50; spectinomycin, 25; and streptomycin, 25.

Transposon mutagenesis of *P. syringae* pv. *glycinea*. Two-parental matings were carried out to introduce the suicide plasmid pCAM140, which harbours a promoterless GUS gene (*uidA*) on the transposon miniTn5 (Wilson *et al.*, 1995), into *P. syringae* pv. *glycinea* PG4180. *E. coli* S17-1 λ -pir containing pCAM140 was used as the donor strain. Plate matings were carried out as described by Bender *et al.* (1991) on King's B medium. Mutants were selected on MG minimal medium supplemented with streptomycin and spectinomycin to select for insertion of the transposon. The ratio of donor to recipient cells was adjusted to obtain transposition frequencies of the order of 10⁻⁵ to 10⁻⁶ with respect to the donor cell number.

DNA procedures. Genomic DNA was isolated from *P. syringae* by established procedures (Staskawicz *et al.*, 1984). Agarose gel electrophoresis, restriction digests, purification of DNA fragments from agarose gels, electroporations, Southern blot hybridizations and small-scale plasmid DNA preparations were performed by standard techniques (Sambrook *et al.*, 1989). For the detection of RFLPs in COR⁻ mutants of PG4180 by Southern blot hybridization, plasmids pMU234 and pMU567 (Ullrich *et al.*, 1994) were used as probes. Subclones were generated in pBluescript SK II(+) (Stratagene). For large-scale preparations, plasmid DNA from *E. coli* was isolated by alkaline lysis and purified with Qiagen columns. The oligonucleotide primer used for sequencing of the DNA adjacent to the miniTn5-*uidA* insertions was 5'-AGATCT-GATCAAGAGACAG-3' and was derived from the I-end sequence of ISS0 (Phadnis & Berg, 1987). Plasmid DNA from *P. syringae* cultures was isolated by the procedure described by Kado & Liu (1981).

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
DH5 α	Used as host in cloning experiments	Stratagene
S17-1 λ -pir	RP4-2 λ -pir, used as delivery host for pCAM140	Simon <i>et al.</i> (1983)
<i>P. syringae</i> pv. <i>glycinea</i>		
PG4180	Wild-type, COR ⁺ GUS ⁻	Bender <i>et al.</i> (1993)
560–632	Sm ^r Sp ^r COR ⁺ transposon mutants of PG4180 showing temperature-dependent GUS expression	This study
615	Sm ^r Sp ^r COR ⁻ transposon mutant of PG4180 with insertion of the miniTn5- <i>uidA</i> upstream of the <i>cmaABT</i> region in the COR biosynthetic gene cluster	This study
Plasmids		
pCAM140	Amp ^r Sm ^r Sp ^r , contains miniTn5- <i>uidA</i> , used for random mutagenesis of PG4180	Wilson <i>et al.</i> (1995)
pBluescript SK II(+)	Amp ^r , cloning vector	Stratagene
pRG960sd	Sm ^r Sp ^r , contains promoterless <i>uidA</i> with start codon and Shine–Dalgarno sequence	Van den Eede <i>et al.</i> (1992)
pRGMU1	Sm ^r Sp ^r , contains <i>cmaABT</i> promoter region on a 3.1 kb <i>Pst</i> I fragment derived from the COR biosynthetic gene cluster in pRG960sd	Ullrich & Bender (1994)
pRGMU3	Sm ^r Sp ^r , contains <i>cmaU</i> promoter region on a 1.4 kb <i>Sal</i> I/ <i>Eco</i> RV fragment derived from the COR gene cluster in pRG960sd	Ullrich & Bender (1994)
pMU234	Tet ^r , contains <i>Sst</i> I fragments 2, 3 and 4 of the COR gene cluster in pLAFR3	Ullrich <i>et al.</i> (1994)
pMU567	Tet ^r , contains <i>Sst</i> I fragments 5, 6 and 7 of the COR gene cluster in pLAFR3	Ullrich <i>et al.</i> (1994)
Designations between p560 and p626	Amp ^r Sm ^r Sp ^r , contain streptomycin-resistance-mediating <i>Sal</i> I fragments from genomic DNA of PG4180 mutants with increased GUS expression at 28 °C in pBluescript SK II(+)	This study
Designations between p561 and p632	Amp ^r Sm ^r Sp ^r , contain streptomycin-resistance-mediating <i>Sal</i> I fragments from genomic DNA of PG4180 mutants with increased GUS expression at 18 °C in pBluescript SK II(+)	This study

DNA sequencing and analysis. Nucleotide sequencing reactions were performed by the dideoxynucleotide method (Sambrook *et al.*, 1989) with the Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham-Buchler) and Cy5-labelled oligonucleotide primers (Pharmacia). Automated DNA sequencing was accomplished using an ALF Express sequencing apparatus (Pharmacia). Additional nucleotide sequencing was carried out commercially (MWG Biotech, Ebersberg, Germany). The single-strand nucleotide sequence data obtained (approximately 300–650 bp) were aligned and processed with the DNASTAR version 4.1 software package (Lasergene, Madison, WI). DNA and protein sequence homology searches of the GenBank, EMBL, PIR and SWISS-PROT databases were performed using the University of Wisconsin Genetics Computer Group (UWCGC) programs BLASTN, BLASTX, FASTEMBL, GAP and BESTFIT.

Assays for GUS activity. GUS activities of transposon mutants were initially screened on MG plates containing 20 μ g of the GUS substrate X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) ml⁻¹ and incubated at 18 or 28 °C for 5–7 d. To standardize the procedure, single mutant colonies were transferred into 96-well microtitre plates filled with MG medium and incubated overnight on a rotary shaker at 100 r.p.m. and 28 °C. A 96-needle metal stamp was used to transfer aliquots of the mutant suspensions onto MG plates containing X-Gluc. Intensities of blue colour formation were visually estimated by three individual researchers in inde-

pendent screenings. Photometric GUS quantification using *p*-nitrophenyl glucuronide as substrate was carried out as described by Wilson *et al.* (1992). Fluorometric analysis of GUS activity (Xiao *et al.*, 1992) was carried out using a Fluorolite-1000 micro-plate reader (Dynatech) and 96-well microtitre plates.

Detection and quantification of COR synthesis. Organic acids were extracted from cell-free bacterial supernatants (1.5 ml) and analysed for the presence of COR using the HPLC method described elsewhere (Palmer & Bender, 1993).

UV sensitivity assay. Strains of *P. syringae* were grown to mid-exponential phase in 5 ml KB medium broth. The cultures were pelleted by centrifugation and resuspended in 5 ml 0.9% PBS (58 mM Na₂HPO₄, 17 mM NaH₂PO₄, 68 mM NaCl, pH 7.4). UV sensitivity assays were performed using the method of Simonson *et al.* (1990), and survivors were enumerated after plating cells on MG agar plates at 28 °C. At least three replicate UV sensitivity assays were performed for each strain.

Plant inoculation experiments. Soybean plants (*Glycine max* cv. Maple Arrow) were grown in a greenhouse at 20–25 °C and 60% humidity, and with a 12 h photoperiod (15000 lux; cold white fluorescent light). Four-week-old soybean plants were sprayed with bacterial cultures grown at 23 °C to an OD₆₀₀ of 1.0 (approx. 1×10^9 c.f.u. ml⁻¹). The

soybean plants were then transferred to a growth chamber (Controlled Environments) at 23 °C and 80% humidity, and with a 12 h photoperiod (15000 lux; cold white fluorescent light). For determination of the *in planta* growth of *P. syringae*, three leaf disks (each with an area of 0.5 cm²) from different plants were removed 7 d post-inoculation, surface-sterilized with 70% ethanol, pooled and homogenized in sterile 0.9% NaCl. Serial dilutions were subsequently plated onto KB agar plates for determination of bacterial numbers (c.f.u. ml⁻¹).

RESULTS AND DISCUSSION

Recently, numerous novel techniques have been developed to quantify global differential gene expression in bacteria *in vitro*. Among those, random insertion of reporter gene fusions, and also differential display methods, subtractive hybridization and DNA array studies have proven to be powerful tools to identify bacterial genetic loci that are differentially expressed upon a given environmental stimulus. Wilson *et al.* (1995) have previously used the transposon miniTn5-*uidA* to study differential gene expression in rhizobia and other Gram-negative bacteria. The present study represents the first approach to broadly identify temperature-responsive genetic loci in a plant-pathogenic bacterium.

Transposon mutagenesis of *P. syringae* pv. *glycinea* PG4180

P. syringae pv. *glycinea* PG4180 was mutagenized by random insertion of the miniTn5-*uidA* transposon (Wilson *et al.*, 1995) following its delivery on suicide plasmid pCAM140 by conjugation. Upon miniTn5-*uidA* insertion, transcriptional fusions could be formed between the promoterless *uidA* gene and *P. syringae* promoters and the *uidA* gene could be expressed, resulting in production of GUS. In total, 7500 individual mutants were obtained, which were subsequently screened for GUS expression on MG agar plates at 18 and 28 °C (see below). The random character of the transposon insertions was verified with respect to the location of the miniTn5-*uidA* by analysing genomic DNA of 72 randomly picked mutants using Southern blot hybridization with pCAM140 as DNA probe (data not shown). Only three mutants showed signals with similar migration patterns, suggesting that the majority of the mutants contained the miniTn5-*uidA* insertion at different genomic locations.

Qualitative determination of temperature-dependent reporter gene expression

To analyse all 7500 transposon mutants for temperature-dependent reporter gene expression, a qualitative GUS assay on X-Gluc-containing MG minimal medium plates was carried out at 18 and 28 °C. Three individual researchers visually estimated development of blue coloration after 5–7 d (Fig. 1). As controls, we included three PG4180 transconjugants on each microtitre plate (marked with open arrows in Fig. 1). One transconjugant harboured plasmid pRG960sd (Van den Eede

Fig. 1. Qualitative screening for thermoresponsive *uidA* expression in 93 transposon mutants of PG4180. Open arrows indicate PG4180 transconjugants harbouring (A) pRG960sd, (B) pRGMU3 or (C) pRGMU1. The filled arrows indicate mutant strains with increased GUS expression at 18 °C (blue) or 28 °C (red). Bacteria were stamped on MG medium plates containing X-Gluc, the substrate for GUS.

et al., 1992), which carries a promoterless *uidA* gene and served as negative control. The other two transconjugants carried plasmids pRGMU1 and pRGMU3 (Ullrich & Bender, 1994), which contained transcriptional fusions of *uidA* to the *cmaABT* upstream region and the *cmaU* promoter region, respectively, from within the COR biosynthetic gene cluster. *cmaABT* is transcribed in a temperature-dependent fashion, whereas the *cmaU* promoter functioned constitutively (Ullrich & Bender, 1994; Budde *et al.*, 1998). All three transconjugants exhibited the expected phenotype (Fig. 1). Blue colour formation varied widely among the 7500 mutants tested, with approximately 50% of the transposon mutants expressing GUS (blue colour) and approximately 50% exhibiting a white colony phenotype, indicating that GUS was not expressed. Only in those mutants that were repeatedly selected by all three researchers was GUS activity considered to be influenced by temperature. Three

hundred and forty-six transposon mutants (4.7% of the total number of mutants) were selected for re-evaluation by the subsequent quantitative GUS assay.

Quantitative measurement of temperature-dependent reporter gene expression

The 346 PG4180 mutants with apparently thermo-responsive GUS phenotypes were tested in a photometric GUS assay following incubation in HSC medium at 18 and 28 °C. The transconjugants PG4180(pRG960sd), PG4180(pRGMU1) and PG4180(pRGMU3) were again included as controls. GUS expression was considered to be thermoresponsive when the ratio of absolute GUS values [expressed as U GUS (mg protein)⁻¹] derived from 18 and 28 °C cultures was greater than 2 (i.e. at least a twofold induction) or less than 0.5. Interestingly, an unambiguous, temperature-dependent *uidA* expression was not found in a large number (206) of the tested mutants (data not shown). The three PG4180 transconjugants used as controls showed the expected phenotypes. Whilst PG4180(pRG960sd) showed no GUS expression in this assay, PG4180(pRGMU1) showed a fivefold-increased GUS expression at 18 °C and transconjugant PG4180(pRGMU3) exhibited a constitutive expression of the reporter gene, as described previously (Ullrich & Bender, 1994). To substantiate the positive results for the remaining 140 transposon mutants, a third screening procedure that involved more elaborate, but also more sensitive fluorometric GUS detection was carried out. Whilst the three controls showed the expected results, only 87 out of the remaining 140 mutants could be confirmed as expressing the *uidA* reporter gene in a temperature-dependent manner. These 87 thermoresponsive mutants represented less than 1.3% of the total number of mutants and less than 2.6% of the GUS-expressing transposon mutants. In summary, by combining a visual test with two subsequent quantitative assays we attempted to lower the likelihood of misinterpretation and subjective errors. The large number of false-positive clones selected upon the initial visual screening demonstrated that quantification of reporter gene expression is an unambiguous pre-requisite for an analysis like this. The three controls used, i.e. a promoterless *uidA* gene on pRG960sd, the 18 °C-inducible *cmA*BT:*uidA* fusion on plasmid pRGMU1, and the constitutively expressed *cmAU*:*uidA* fusion, all in PG4180, showed the expected phenotypes in both the qualitative and the subsequent quantitative assays. This result validates the chosen three-step screening procedure.

Characterization of individual transposon mutants of PG4180

Southern blot experiments with a 2.0 kb *NotI* fragment of plasmid pCAM140 carrying the *uidA* gene as the DNA probe and genomic DNA of all 87 mutants treated with restriction enzyme *SalI* were carried out (data not shown). This experiment aimed to identify duplicates among the selected mutants since the signals obtained should migrate in a manner characteristic for each

Fig. 2. Quantification of GUS expression in thermoresponsive transposon mutants expressed as the ratio of GUS expression values at 18 and 28 °C. Values above 0 indicate increased GUS expression at 18 °C (plotted highest to lowest) whereas values below 0 indicate increased expression at 28 °C (plotted lowest to highest). Bars represent mean values from 61 mutants in three experiments, each with two replicates.

insertion locus. Interestingly, signals in genomic DNA of 26 mutants occurred simultaneously in two individually picked mutants, suggesting that the transposon insertion loci of these mutants were identical. Therefore, we limited all further experiments to the remaining 61 mutants with individual genotypes. From those, 39 transposon mutants showed an increased GUS expression at 18 °C and 22 exhibited a stronger *uidA* transcription at 28 °C. As depicted in Fig. 2 the level of temperature induction expressed as the ratio of GUS activities at 18 versus 28 °C or vice versa varied between 2- and 40-fold. The actual GUS expression values ranged between 15 and 2500 U GUS (mg protein)⁻¹ (data not shown). On one hand, the strong variability in total GUS values suggested a high diversity in promoter strengths. On the other hand, this result might indicate that the transposon inserted in variable proximity to the respective promoters, giving rise to higher or lower levels of reporter gene expression.

Identification of transposon mutants impaired in COR biosynthesis

COR biosynthetic genes were previously reported to be transcribed at maximal levels at 18 °C and at low basal expression levels at 28 °C (Ullrich & Bender, 1994; Ullrich *et al.*, 1995; Liyanage *et al.*, 1995). Consequently, supernatants of all 39 mutants with increased GUS expression at 18 °C were analysed for the presence of COR by organic acid extraction and HPLC. Twenty-seven of these mutants were impaired in COR biosynthesis, indicating that the transposon might have inserted in the 32.8 kb COR gene cluster (data not shown). Southern blot hybridizations with specific probes spanning the entire COR gene cluster (see Methods) and subsequent determination of the DNA flanking the miniTn5-*uidA* insertions for some of these mutants confirmed these results (data not shown). In summary, our results demonstrated that the applied screening procedure was suitable and highly efficient for

Table 2. Similarities of gene products of PG4180 mutants with known gene products and predicted function of thermo-responsive loci

Mutant	% Amino acid sequence identity (% similarity), protein, organism [accession no.]*	Predicted function or property	GUS expression [U (mg protein) ⁻¹] [†]	
			18 °C	28 °C
Mutants with increased GUS expression at 28 °C				
560	42 (53), DFR, <i>Arabidopsis thaliana</i> [S07463]	Dihydroflavanol 4-reductase; flavanoid biosynthesis	416	954
563	35 (62), Hrl53, <i>Rhizobium leguminosarum</i> [P02348]	HU-like DNA binding protein	50	396
564‡	39 (58), Eae, <i>Citrobacter freundii</i> [I40705]	Intimin, attaching and effacing locus, bacterial adhesin, host cell contact	201	634
590	40 (48), AspA, <i>Aeromonas salmonicida</i> [S26691]	Serine protease, subtilase family of proteases	96	363
591	74 (87), VacC, <i>Shigella flexneri</i> [Q54177]	tRNA-guanine transglycosylase, tRNA modification	12	65
593	50 (60), WbpN, <i>P. aeruginosa</i> [U50396]	B-band lipopolysacchide biosynthesis, homocitrate synthase	26	220
596	43 (64), TraL/KorB, <i>Agrobacterium tumefaciens</i> [X16905]	T-DNA transfer, conjugal plasmid transfer	123	481
599	81 (92) MucD, <i>P. aeruginosa</i> [U30799]	Periplasmic serine protease, HtrA-like, modulatory function in alginate synthesis	692	1805
605	100, Ebr (E1), <i>E. coli</i> [P14502] and QacE, <i>P. aeruginosa</i> [M73819]	Small multi-drug transporter, ethidium bromide resistance	96	432
608‡	38 (60), RfbC, <i>Riftia pachyptila</i> endosymbiont [AF105060]	O-antigen LPS biosynthesis	268	804
613	39 (68), RulAB, <i>P. syringae</i> [JC5162]	Resistance to UV light, UmuD homologue	34	140
617	45 (59), ORF1, <i>Erwinia chrysanthemi</i> [L39897]	Cyclase involved in polyketide synthesis, downstream of <i>hrpN</i>	45	186
618	44 (48), <i>uvrA</i> region, 119.5 kDa hypothetical protein, <i>Micrococcus luteus</i> [JQ0405]	DNA damage and UV mutagenesis repair system	29	72
626	93 (94), BraF, <i>P. aeruginosa</i> [P21629]	High-affinity branched-chain amino acid permease	34	510
572, 573, 578, 579, 592, 597, 602, 612	No significant similarities to any database entries	Unknown		
Mutants with increased GUS expression at 18 °C				
561	53 (58), AlgE2, <i>Azotobacter vinelandii</i> [S77626]	Mannuronan C-5-epimerase involved in alginate biosynthesis	406	81
562	54 (68), IS870 element, <i>A. tumefaciens</i> [B36919]	Insertion element IS870, transposase	120	45
568	79 (92), FdhA, <i>E. coli</i> [D38504]	Formaldehyde dehydrogenase, similar to alcohol dehydrogenases	131	62
570	36 (44), Y4mP, <i>Rhizobium</i> sp. NGR234 [P55575]	Short-chain-type dehydrogenase (putative)	180	15
574	100, HrpT, <i>P. syringae</i> [A45243]	Type III secretion apparatus, hypersensitive response and pathogenicity	332	128
601	72 (77), RepA, <i>P. syringae</i> [AJ222648]	Plasmid replication protein	308	57
615‡	100 <i>cmaABT</i> upstream region <i>P. syringae</i> [U14657]	COR biosynthesis	695	84
632	31 (35), hypothetical protein from replication region of plasmid R91-5, <i>P. aeruginosa</i> [S21306]	Plasmid R91-5 replication	582	206
565, 570, 580, 586, 627	No significant similarities to any database entries	Unknown		

* Bacterial proteins with greatest amino acid sequence identity to the predicted protein sequences. GenBank/EMBL/PIR/SWISS-PROT accession numbers are shown in square brackets.

† Data represent mean values of three replicate experiments.

‡ PG4180 mutants with impaired virulence on soybean plants.

the identification of known thermoresponsive genetic loci of PG4180. The relatively large number of COR-defective mutants amongst the mutants with induced GUS expression at 18 °C could be explained in part by the size of the COR biosynthetic gene cluster (32.8 kb) or by the possibility of multiple copies of plasmid p4180A harbouring this gene cluster within the cell.

Transposon mutants with increased reporter gene expression at 28 °C

Following *SalI* digestion of genomic DNA, the genetic loci affected by the miniTn5-*uidA* insertion in the 22 mutants with induced *uidA* expression at 28 °C were shotgun-cloned into *SalI*-treated pBluescript SK II(+). Transformants were screened for resistance to ampicillin, streptomycin and spectinomycin, and their plasmid DNA was subsequently digested with *SalI* to verify the cloning results. The subcloned miniTn5-*uidA* insertions were given designations between p560 and p626 according to the corresponding mutant numbers (Table 2). Subsequently, the DNA adjacent to the I-end of the insertion sites was sequenced. The nucleotide sequences were compared with database entries and the results are summarized in Table 2. Unfortunately, the insert DNA derived from eight of these mutants showed no significant sequence similarities to any database entries.

Mutants 593 and 608 contained transposon insertions in genes homologous to loci involved in LPS synthesis (Burrows *et al.*, 1996) (Table 2), which may be implicated in virulence mechanisms in plant pathogens such as *P. syringae* (Graham *et al.*, 1977). Newman *et al.* (1997) showed that treatment with the lipid A fraction of bacterial LPS prior to inoculation could prevent the recognition of pathogens by resistant plants. Sequeira (1985) hypothesized that LPS might be involved in initial attachment of bacterial cells to plant cell walls.

Regulatory genes might have been affected in mutants 563, 591 and 599. The insertion loci of these three mutants showed similarities, respectively, to a HU-like DNA-binding protein of *Rhizobium leguminosarum* (Khanaka *et al.*, 1985); to VacC, involved in virulence regulation of *Shigella flexneri* (Durand *et al.*, 1994); and to MucD, a regulatory periplasmic serine protease involved in alginate biosynthesis of *Azotobacter vinelandii* and *Pseudomonas aeruginosa* (Martinez-Salvazar *et al.*, 1996; Ertesvag *et al.*, 1995). With respect to the latter mutant, *P. syringae* is also known to produce this exopolysaccharide and alginate synthesis was shown to be controlled by temperature, with increased *algD* transcription at 28 °C (Peñaloza-Vázquez *et al.*, 1997).

Three mutants, 605, 596 and 626, apparently harboured miniTn5-*uidA* insertions in genes homologous to those, respectively, of a small multi-drug transporter (Sundstroem *et al.*, 1988); of the TraL/KorB locus, required for T-DNA and plasmid DNA transfer in *Agrobacterium tumefaciens* and *E. coli* (More *et al.*, 1996; Winans & Walker, 1985); and of a high-affinity

Fig. 3. Analysis by agarose gel electrophoresis of undigested plasmid DNA of PG4180 mutants with insertions in putative plasmid-borne loci. The white arrow indicates the non-mutagenized plasmid p4180E and the black arrow indicates the mutagenized plasmid p4180E, which is shifted in electrophoretic mobility by 4.7 kb. Lanes: 1, 596; 2, 632; 3, 601; 4, PG4180(pRGMU3); M, molecular size markers.

branched-chain amino acid permease from *P. aeruginosa* (Hoshino & Kose, 1990). All three loci may be involved in transport or secretion processes. To find out whether the *traL/korB* locus in mutant 596 was plasmid-borne, its plasmid profile was analysed for an alteration in electrophoretic mobility due to the miniTn5-*uidA* insertion (Fig. 3, lane 1). Mutant 596 showed a shift in the band representing the 45 kb indigenous plasmid p4180E, raising the interesting hypothesis that this plasmid might harbour a conjugal *traL* locus. Subsequently, Southern blot analysis was carried out using the insert DNA of p596 as DNA probe and undigested plasmid DNA of mutant 596 and the PG4180 wild-type (data not shown), indicating that a *traL/korB*-like locus is only present on the 45 kb plasmid p4180E but not on any of the other indigenous plasmids of PG4180. In a subsequent study, we have identified a total of 12 *tra*-like genes in close proximity to the *traL*-like locus of p4180E, suggesting that this plasmid might harbour a *tra* operon (K. Leykauf & M. S. Ullrich, unpublished observation). Conjugation experiments are currently being conducted in our laboratory to test whether PG4180 plasmids are transferred in a temperature-dependent manner. Conjugal plasmid transfer and plasmid replication in different organisms has previously been shown to undergo temperature regulation (Fullner & Nester, 1996; Fernandez-Tresguerres *et al.*, 1995; Tietze & Tschape, 1994; Banta *et al.*, 1998).

Two additional loci were similar to biosynthetic genes, a plant dihydroflavanol 4-reductase of *Arabidopsis*

Fig. 4. Analysis of survival of PG4180 (○), and mutants 613 (●) and 618 (□) after exposure to UV irradiation. Values are means of three experiments with two replicates per strain.

thaliana and garden petunia (Beld *et al.*, 1989) and a putative cyclase gene designated ORF1 and possibly involved in polyketide synthesis from *Erwinia chrysanthemi* (Kim *et al.*, 1998). Mutant 564 might carry the reporter gene in a locus encoding a bacterial adhesin-like protein which has not explicitly been reported in plant pathogens thus far, but is an important virulence determinant in animal pathogens (Schauer & Falkow, 1993). Furthermore, the subcloned locus of mutant 590 resembled that of a serine protease gene in *Aeromonas salmonicida* (Whitby *et al.*, 1992).

Interestingly, the insertion loci of mutants 613 and 618 showed sequence homologies, respectively, to the UV resistance locus *rulAB* of *P. syringae* (Sundin *et al.*, 1996) and to the *uvrA* region of *Micrococcus luteus* (Shiota & Nakayama, 1989), which both may represent stress-inducible genetic loci. To test this, both mutants were screened for impaired resistance to UV irradiation (Fig. 4). Mutant 613 was indeed more susceptible to UV damage than the PG4180 parental strain. In contrast, the other mutant was not affected in its ability to withstand UV irradiation under the tested conditions. It could be speculated that expression of UV tolerance genes is more important when the bacteria grow epiphytically on the UV-exposed, warmer surface of plant leaves (Beattie & Lindow, 1999) than when the bacteria enter the plant apoplast to become virulent. To determine if strain PG4180 shows a temperature-dependent UV tolerance, cells were incubated in KB liquid medium at 18 and 28 °C and dilution series of each culture were plated onto MG agar plates, which were subsequently exposed to UV irradiation of 30 J m⁻². Bacterial survival rates were 1–2 % for cells pre-incubated at 28 °C and less than 0.2 % for the samples pre-incubated at 18 °C, suggesting that PG4180 is more sensitive to UV damage at the lower temperature. This result is in line with an increased expression of the *rulAB* locus at 28 °C.

Characterization of phytotoxin-producing PG4180 mutants with increased GUS expression levels at 18 °C

From a total of 39 PG4180 mutants with increased GUS expression at 18 °C, 12 mutants produced COR and therefore were considered to possess transcriptional fusions of the *uidA* gene with genetic loci outside the COR gene cluster. Following the *SalI*-mediated subcloning from genomic DNA and restriction mapping, the respective clones were given a designation between p561 and p632 according to the corresponding mutant numbers (Table 2). Nucleotide sequences directly adjacent to the I-end of miniTn5-*uidA* were determined and compared with database entries. Results are summarized in Table 2 and indicate that for five mutants no significant sequence similarities were obtained.

The genetic loci p561, p568 and p570 showed similarity in their predicted amino acid sequences to the biosynthetic proteins mannuronan C-5-epimerase (AlgE2) of *Azotobacter vinelandii* (Ertesvag *et al.*, 1995), to formaldehyde dehydrogenase of *E. coli* (Gutheil *et al.*, 1992) and to a putative short-chain dehydrogenase from *Rhizobium* strain NGR234 (Freiberg *et al.*, 1997). The result for mutant 561, which carries the reporter gene insertion in an *algE*-like locus (Table 2) is in stark contrast to that of mutant 599, which has an insertion in a *mucD*-like locus and shows increased GUS expression at 28 °C. If *mucD* of PG4180 were part of the *algT* operon, one would expect that the *algE*-like locus would be expressed preferentially at 28 °C. Transcription of *algE* in *P. aeruginosa* and *Azotobacter vinelandii* was previously shown to be under the control of AlgT (Ertesvag *et al.*, 1995; Martinez-Salvazar *et al.*, 1996). Since copper ions induce alginate biosynthesis in *P. syringae* (Kidambi *et al.*, 1995), we subsequently tested the alginate production of mutants 561 and 599 on MG agar plates supplemented with 250 µg cupric sulfate ml⁻¹ and compared it to the phenotype of the wild-type. No obvious differences in exopolysaccharide production were observed (data not shown). This result suggested that the *algE*-like locus affected in mutant 561 might not be involved in alginate biosynthesis.

Mutant 574 carried a transposon insertion in *hrpT*, a gene involved in assembly of the type III *hrp* (hypersensitive reaction and pathogenicity) protein secretion apparatus. This secretion system is required for pathogenicity and induction of the plant-borne hypersensitive response (HR) caused by *P. syringae* (Deng *et al.*, 1998). When tested for its HR phenotype, this mutant still elicited a HR on tobacco plants (data not shown). This result might be due to the fact that the transposon had inserted at the 3' end of *hrpT*, thus not completely blocking its expression. Since *hrpT* is located at the 3' end of the respective *hrpFGCTV* operon (Deng *et al.*, 1998) the miniTn5-*uidA* insertion might not have caused any polar effects on the transcription of the other *hrp* genes. Regardless, this finding is interesting since *hrp* gene expression in *Erwinia amylovora* has previously been shown to be thermoresponsive (Wei *et al.*, 1992).

and the *hrp*-mediated protein secretion mechanism in *P. syringae* had unambiguously been demonstrated to be temperature-dependent with maximal activity at 18–20 °C (Van Dijk *et al.*, 1999).

Two additional loci, designated p601 and p632 (Table 2), showed relatedness, respectively, to the plasmid replication protein RepA of *P. syringae* pv. *phaseolicola* (Gibbon *et al.*, 1999) and to the replication region of the *P. aeruginosa* plasmid R91-5 (Moore & Krishnapillai, 1982), suggesting plasmid-borne locations. Agarose gel electrophoresis of undigested plasmid preparations from mutants 632, 601 and PG4180(pRGMU3) (Fig. 3, lanes 2, 3 and 4) demonstrated that mutant 601 showed a shifted band representing the 45 kb plasmid p4180E. This indicated that the miniTn5-*uidA* had inserted in this plasmid. In contrast, the plasmid profile of mutant 632 did not differ from that of PG4180(pRGMU3), suggesting that the sequence similarity to the replication region of plasmid R91-5 was misleading (Fig. 3).

The cloned DNA of mutant 562 showed strong sequence similarity to IS870, an insertion element frequently found in *P. syringae* and other plant pathogens (Fournier *et al.*, 1993). Turner *et al.* (1990) reported on the temperature sensitivity of transposition of class II transposons with optimal levels of transposition at room temperature or 30 °C versus 37–42 °C. Our result might reflect that transposition of IS870 could also be more pronounced at lower temperatures.

Effects of transposon mutations on growth rates *in vitro* and *in planta*

To determine whether mutations in the identified genetic loci affected the *in vitro* growth of *P. syringae*, we compared the generation times of the PG4180 wild-type and 34 distinct mutants representing all identified genetic loci (12 with increased GUS expression 18 °C and 22 with increased GUS expression at 28 °C) in HSC medium at 18 and 28 °C. The bacterial growth of duplicate cultures was measured photometrically. No significant differences between the wild-type and any of the mutants were observed (data not shown), suggesting that the transposon insertions had no effect on the *in vitro* growth of the bacteria.

Furthermore, plant inoculation experiments were carried out with all 34 PG4180 mutants, one of the COR[−] mutants (designated 615) and the wild-type strain. Following spray-inoculation of bacteria grown at 23 °C onto soybean plants (subsequently kept at 23 °C), we monitored disease development and enumerated the bacterial populations inside the plant tissue after 7 d. Most of the mutants tested were as virulent as the wild-type and reached population densities of approximately $3\text{--}7 \times 10^7$ c.f.u. cm^{−2}. In contrast, three mutants (615, 564 and 608) showed a decreased development of bacterial blight symptoms on the host plant and lowered bacterial multiplication ($1\text{--}4 \times 10^6$ c.f.u. cm^{−2}) inside the plant tissue, indicating that their transposon insertions interfered with the virulence of the bacteria. Whilst 615 was the COR[−] mutant tested, thus confirming

previous findings on the role of COR as a virulence factor (Bender *et al.*, 1987; Mittal & Davis, 1995; Tamura *et al.*, 1998; Budde & Ullrich, 2000), the insertion locus of 564 showed similarities to bacterial adhesins and that of 608 was similar to LPS biosynthetic loci. This result hints at a potential role of both loci in virulence associated processes. The reason why the latter two mutants – although impaired in virulence – showed an increased GUS expression at 28 °C, a temperature non-conducive for the initial establishment of a successful invasion process for PG4180 (Dunleavy, 1988; Budde & Ullrich, 2000) remains obscure. It could be speculated that the two affected genetic loci might be required for later steps of the disease development during which low temperature might not be a triggering key factor anymore. Future experiments in our laboratory will aim at unravelling their respective functions in the plant–microbe interaction.

Time-dependence of temperature induction among *P. syringae* mutants

We were interested in determining the time span that is necessary to induce reporter gene expression in the identified PG4180 mutants. Cultures of the 34 thermo-responsive mutants were incubated at the respective non-conductive temperature until they reached an OD₆₀₀ of 1.0. Subsequently, the bacterial cultures were shifted to the growth temperature that induced GUS expression and were incubated for an additional 24 h. Samples were taken at intervals of 1–2 h for determination of GUS expression levels and results for all mutants were plotted against time. Results for some representative mutants are depicted in Fig. 5. In general, there was a considerable lag phase for GUS induction (2–6 h), indicating that extended time spans were required for the bacteria to adjust to the new temperature regime before GUS expression was initiated. As a control, we included incubation of PG4180(pRGMU3), which harbours a constitutively expressed *cmaU:uidA* fusion (Ullrich & Bender, 1994). This transconjugant did not show a temperature-dependent induction of GUS synthesis. Its initial GUS values at the time of temperature shift from 18 to 28 °C or vice versa were 154 ± 20 and 210 ± 14 U GUS (mg protein)^{−1}, respectively. Upon temperature shifts, GUS values for this transconjugant reached 243 ± 31 and 279 ± 25 U GUS (mg protein)^{−1}, respectively, after 15 h incubation. This is in line with previously reported results (Ullrich & Bender, 1994). All identified thermoresponsive fusions showed a slow and steady increase in reporter gene expression once the bacteria were shifted to the inducing temperature. This indicates that the initial screening procedure had biased temperature-shock-dependent promoters. Moreover, these results suggested that the general type of thermoregulation for most of these genetic loci might be similar. If at least some of the identified genetic loci are part of a regulon, subsequent mutagenesis could be used to identify regulatory components that function at higher hierarchical levels of thermoregulation in *P. syringae*.

Fig. 5. Time-dependent GUS expression in transposon mutants following a temperature shift. Bacteria were grown to an OD of 1.0 ($t = 0$ h) at the non-inductive temperature, incubated for an additional hour and then shifted (arrow) to the GUS-expression-inducing temperature. Values are means of three replicates per strain. (a) Shift from 28 to 18 °C. ♦, 561; □, 615; ▲, 570; ●, 601. (b) Shift from 18 to 28 °C. ♦, 563; □, 596; ▲, 613; ●, 593.

In summary, the genetic loci affected in the identified COR⁺ mutants could be categorized into five groups: (i) those with putative biosynthetic functions; (ii) those with a regulatory function; (iii) those with functions in secretion and transport processes; (iv) those putatively functioning in cell wall synthesis, assembly of cell appendages, and plasmid replication and transfer; and (v) those associated with a stress response.

An increase in reporter gene expression at a given temperature could also be interpreted as a repression of transcription at the other temperature. The data and the methods used herein did not allow us to discriminate unambiguously between these possibilities. Use of unstable – and hence temperature-independent – reporter genes fused to the respective loci will answer this question in future studies. The results of this work have initiated a number of subsequent studies that ultimately will deepen our understanding of the molecular basis of thermoregulation in plant-pathogenic bacteria.

ACKNOWLEDGEMENTS

The authors thank G. W. Sundin and C. L. Bender for stimulating discussions. Special thanks to Bianca Pohlack and Sabine Wehl for excellent technical support. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Max-Planck-Society.

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Received 7 June 2000; revised 9 July 2000; accepted 24 July 2000.